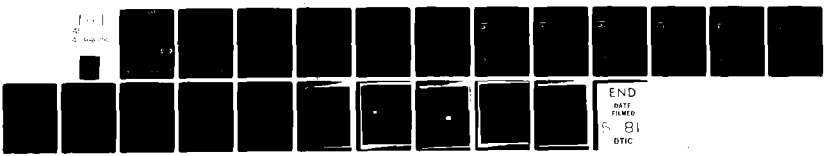
AD-A098 075 INDIANA UNIV MEDICAL CENTER INDIANAPOLIS DEPT OF SURGERY F/G 6/5
SELECTIVE IMMUNOSUPPRESSION BY ANTI-IDIOTYPIC ANTIBODY IN THE N--ETC(U)
APR 81 S B LEAPMAN N00014-78-C-0412

UNCLASSIFIED NL



LEVEL II

13

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER N00014-78-C-0412	2. GOVT ACCESSION NO. AD-A098075	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) SELECTIVE IMMUNOSUPPRESSION BY ANTI-IDIOTYPIC ANTIBODY IN THE NON-HUMAN PRIMATE		5. TYPE OF REPORT & PERIOD COVERED Final Report August 1978 - December 1980
		6. PERFORMING ORG. REPORT NUMBER Task No NR 207-112
7. AUTHOR(s) Stephen B. Leapman, M.D.		8. CONTRACT OR GRANT NUMBER(s) N00014-78-C-0412
9. PERFORMING ORGANIZATION NAME AND ADDRESS Indiana University Medical Center Department of Surgery Indianapolis, Indiana 46202		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS Work unit number N00014-78-C-0412
11. CONTROLLING OFFICE NAME AND ADDRESS W.S. Johnson Director of Research Management Indiana University Foundation		12. REPORT DATE 15 April 1981
		13. NUMBER OF PAGES 5
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Distribution of this report is unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

A

DTIC ELECTE APR 23 1981 S D F

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Antibody
Anti-idiotypic antibody
Cell surface receptors

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

An attempt to raise an anti-idiotypic antibody in the stump-tailed macaque, M. speciosa, was unsuccessful. From these efforts, however, data regarding lymphocyte responsitivity to various stimulators and immunosuppressants was obtained. This data is noted in the final report and in the enclosed scientific publications.

A

New 412303

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE S/N 0102-LF-014-6601

81 4 22 173

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

AD A098075

DTIC FILE COPY

OFFICE OF NAVAL RESEARCH

CONTRACT N00014-78-C-0412

Task No. NR 207-112

FINAL REPORT

SELECTIVE IMMUNOSUPPRESSION BY ANTI-IDIOTYPIC ANTIBODY IN THE NON-HUMAN PRIMATE

by

Stephen B. Leapman, M.D.
Indiana University Medical Center
Department of Surgery
Indianapolis, Indiana 46202

15 April 1981

Distribution of this report is unlimited.

1. Since the inception of this contract in August 1978, research activities have been directed toward the development and definition of an anti-idiotypic antibody (AIA) in the outbred primate, M. speciosa, the stump-tailed macaque. Prior work at the Naval Medical Research Institute had demonstrated that such an antibody could be raised in a chimpanzee model. Our purpose was to develop this antibody in a small, less expensive macaque model. In addition, this model gave us an opportunity to (a) study the effect of various immunosuppressive agents on cell surface receptors and (b) aid in our understanding of immune regulatory mechanisms.

2. As noted in the first 6 month report, equipment and supplies necessary for cellular culture techniques and immunology cellular assay systems were developed. By February 1979, the laboratory was quite functional and data concerning the lymphocyte properties of M. speciosa were being defined. A careful kinetic examination of the cellular immune response in this species to both alloantigenic and mitogenic stimuli was performed. Results of these studies are noted below:

a. Mitogens - mitogenic concentrations ranged through 3 log increments. Mitogen studies included PHA(M) 0.01-5%, Concanavalin-A 1-1000μgm/ml, Pokeweed 0.1-50μgm/ml and LPS (E. coli) 0.1-100μgm/ml. Peak CPM's and time of maximum response are as follows:

MITOGENS	CPM x 10^3 ± S.E.	DAY
PHA 1%	190 ± 2.5	4.5
Con-A 25μgm/ml	360 ± 4.1	4
Pokeweed 0.5μgm/ml	154 ± 6.3	4.5
LPS 100μgm/ml	15 ± 1.2	4

b. Alloantigens (MLR) - alloantigenic responses were studied in mixed lymphocyte culture. Peak responses were noted on Day 5 ± 0.5 with 23,794 ± 2,569 cpm.

c. Primed Cell Stimulation - primed cells were prepared as described in the original contract proposal. Briefly, cells from responder monkey (A) were co-cultured with irradiated cells from the stimulator monkey (Bx) for 10 days. On the 10th day these cells were harvested and re-stimulated with the original cell (Bx). As noted below we clearly demonstrate the ability of these "primed" cells to respond in a secondary MLC.

	PRIMED RESPONDER cpm ± S.E.	FRESH UNPRIMED RESPONDER cpm ± S.E.	FROZEN UNPRIMED RESPONDER cpm ± S.E.
Day 2	16,322 ± 1,534	7,432 ± 306	3,197 ± 424
Day 3	8,717 ± 374	6,175 ± 195	5,603 ± 623
Day 4		13,235 ± 2,200	2,419 ± 502
Day 5		26,149 ± 1,649	12,908 ± 636

d. Despite the ability to produce primed cells responsive to specific alloantigens, we were unable to produce the quantity ($>10^9$) of these cells for immunization and subsequent boosts. This was a critical limiting step in the experiments since we were never able to harvest enough cells for immunization in this small macaque model. Unlike the chimpanzee where we could use a cell-separator and harvest large quantities of lymphocytes, the selective immunosuppression by anti-idiotypic antibody in the non-human primate stump-tail produced responsive but limited numbers of cells. The result was that no anti-idiotypic antibody was produced.

3. In Vitro Effects of Pharmacologic Agents on Lymphocyte Subpopulation:

Because this study involved the production of specifically primed lymphocytes, experiments were performed to examine the effect of various immunopharmacologic agents on primed cells and other lymphocyte subpopulations. The results of these studies have been published under this contract in separate journals and the results presented at two international congresses and one national meeting. (See Publications.)

Our studies provide evidence that lymphocytes primed against specific alloantigenic cells in the presence of the potent immunosuppressive agent, Cyclosporin-A, lost their ability to respond in an accelerated manner to the same primary alloantigens. A commonly used immunosuppressive agent, hydrocortisone, had no effect on the response of the primed cell in secondary cultures. The experiments suggest that the constant presence of the compound, Cyclosporin-A, may interfere with cell surface receptors in the recognition phase of the immune response. This action may be responsible for a portion of its immunosuppressive actions. Suppressor cells, however, do not seem to be effected by Cyclosporin-A, neither in the generation phase or in the function. This drug did not, however, induce the formation of suppressor cells.

These pilot studies have helped clarify the mechanism of this immunosuppressive agent.

PUBLICATION INDEX

PUBLICATIONS:

1. Leapman, S.B.; Filo, R.S.; Smith, E.J.; and Smith, P.G.: Effects of Cyclosporin-A on the Generation of Memory Cells *In Vitro*. Transplant Proc 12:246-251, 1980.

2. Leapman, S.B.; Filo, R.S.; Smith, E.J.; and Smith, P.G.: *In Vitro* Effects of Cyclosporin-A on Lymphocyte Subpopulations. I. Suppressor cell Sparing by Cyclosporin-A. Transplantation 30:404-408, 1980.

3. Leapman, S.B.; Filo, R.S.; Smith, E.J.; and Smith, P.G.: Differential Effects of Cyclosporin-A on Lymphocyte Subpopulations. Transplant Proc 13: 405-409, 1981.

PRESENTATIONS:

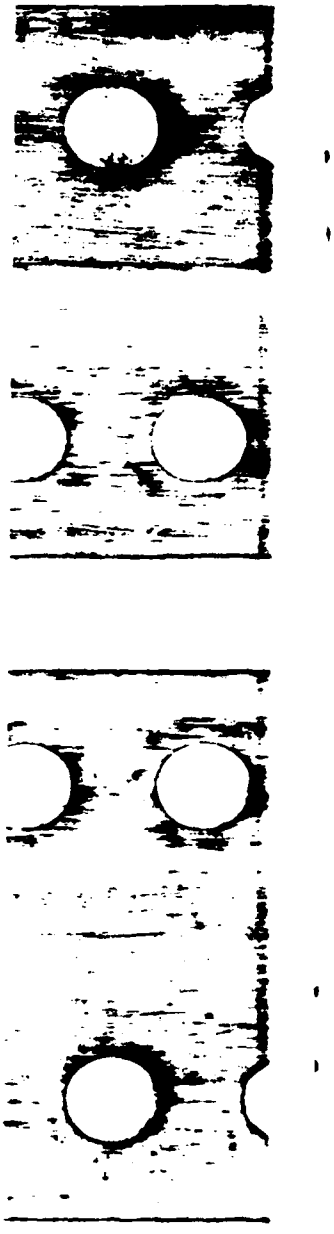
1. Leapman, S.B.: Effects of Cyclosporin-A on the Generation of Memory Cells *In Vitro*. International Symposium on Pharm. Immunosuppression in Organ Transplantation, September 1979, Cardiff, Wales.

2. Leapman, S.B.; Filo, R.S.; Smith, E.J.; and Smith, P.G.: Differential Effects of Cyclosporin-A on Lymphocyte Subpopulations. Poster Session, VIIIth International Congress of the Transplantation Society, Boston, Massachusetts, June 1980.

3. Leapman, S.B.; Filo, R.S.; Smith, E.J.; and Smith, P.G.: Differential Effects of Cyclosporin-A on Lymphocyte Subpopulations. Presented to the American Society of Transplant Surgeons, Chicago, Illinois, May 1980.

Effects of Cyclosporin A on the Generation of Primed Lymphocytes In Vitro

S. B. Leapman, R. S. Filo, E. J. Smith, and P. G. Smith



CYCLOSPORIN A (CY-A), a new antilymphocytic drug, has been described as a potent immunosuppressive agent.[1-3] This compound has been studied in multiple species with various experimental models, yet the mechanisms of its immunosuppressive actions remain unknown. In vivo, CY-A-treated rats have had successful engraftment of bone marrow across AgB-incompatible barriers without the development of graft-versus-host disease.[4] In man, the drug has modified the acute skin reactions commonly seen in graft-versus-host disease.[5] Survival of renal allografts in mongrel dogs has been prolonged by CY-A treatment. In clinical trials with CY-A, Calne et al. have reported some success in patients that have received renal allografts.[6] Jamieson and colleagues[7] found that CY-A without additional immunosuppressants was insufficient to ensure long-term allograft survival in a primate cardiac model. They also noted that short-term administration of the drug with subsequent discontinuance did not permit long-term graft survival. These results are contrary to those reported by Green and Allison.[2] They found that CY-A alone was able to induce long-term renal allograft survival in rabbits and that the animals became tolerant to other organ transplants from the original donor, but not from third-party donors.[8] Furthermore, the tolerance persisted in their animals even after all immunosuppressive therapy had been withdrawn (up to 70 days earlier). Dunn, White, and Wade[9] reported similar results, but indicated less specificity in that 66% of third-party donor allografts survived for prolonged periods even after the withdrawal of CY-A.

Experiments have also been performed in vitro to clarify the mechanisms of this drug. Proliferation of porcine lymphocytes were inhibited with CY-A in a dose-dependent manner when challenged by phytohemagglutinin (PHA) or alloantigenic stimulation. Inhibition was significantly higher if the drug was added at the beginning or soon after the initiation of the culture, but not at the end of the culture period. T-cell proliferation was inhibited with 5–100 times less drug than was B-cell proliferation.[10] Leoni and colleagues[11] studied the response of human lymphocytes in culture to CY-A and reported that this drug inhibits blastogenesis in a dose-dependent manner when cells were stimulated by PHA, conconavalin A, or pokeweed mitogen. Lymphoblasts were the target of CY-A activity and resting cells were not effected.

In order to further elucidate the antilymphocytic mechanisms of CY-A in vitro, a model using normal human lymphocytes was designed to answer the following questions: (1) Will initial CY-A treatment affect the memory of lymphocytes primed to specific alloantigens when challenged by the alloantigens in secondary cultures? (2) Will those same cells be responsive in secondary cultures to third-party alloantigens? (3) Does CY-A have different effects on primed lymphocytes than hydrocortisone (HC), a known immunosuppressant? The results of these experiments form the basis of this report.

MATERIALS AND METHODS

Preparation of Primed Lymphocytes

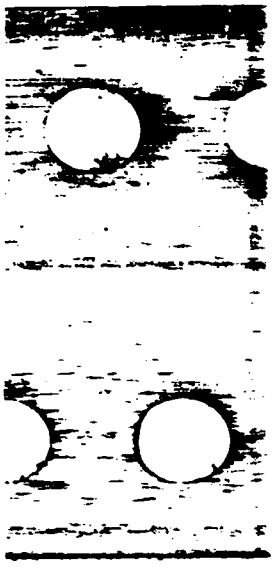
Peripheral blood was donated by healthy volunteers. Mononuclear cells were separated using a Ficoll-Hypa-

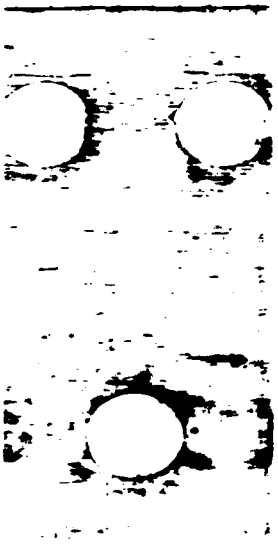
From the Departments of Surgery (Transplantation) and Medicine (Nephrology), Indiana University Medical Center, Indianapolis, Ind.

Supported by ONR Contract N-00014-78-C-0412.

Reprint requests should be addressed to S. B. Leapman, M.D., Departments of Surgery and Medicine, Indiana University Medical Center, Indianapolis, Ind. 46202.

0041-1345/80/1202-0005$01.00/0





que discontinuous gradient procedure (specific gravity 1.078). Lymphocytes were primed in vitro by culturing 16×10^6 cells from responder "A" with 16×10^6 (x-irradiated, 2500 rads) from stimulator "B." Concentrations of responder and stimulator cells were each maintained at 4×10^6/ml with the appropriate RPMI 1640 culture media as described below. Cultures were incubated in 25 sq cm upright flasks at 37°C in a 5% CO_2 humidified atmosphere for 10 days. After the fourth day of incubation, fresh medium was added to the cultures at 2-3-day intervals. Primed cells were harvested on the tenth day, washed with medium, layered over fetal calf serum to remove debris, and then washed again. Cells were counted and prepared for secondary phase cultures.

Preparation of Media

Control media Four separate culture media (2 controls, 1 cyclosporin A, and 1 hydrocortisone) were used in the experiment. Control media were prepared as RPMI 1640 with Hepes (25 m*M*), bicarbonate (2 g/liter), L-glutamine (2m*M* liter), gentamycin (80 mg/liter), and 10% pooled AB human plasma. Because dimethylsulfoxide (DMSO) was used as a solvent for the CY-A, it was necessary to use a control for this agent. Therefore DMSO, 1% v/v, was added to the above RPMI 1640 media, and this preparation served as the DMSO control medium.

Cyclosporin A media (Sandoz, Ltd., Basel, Switzerland) CY-A medium was prepared by the addition of 1 ml of DMSO to 100 μg of CY-A. This was slowly added to the CY-A medium with constant stirring. Three CY-A concentrations, 1.0, 0.5, and 0.1 μg/ml, were used during the priming phase of the experiment. All dilutions of CY-A media were made with 1% DMSO control medium.

Hydrocortisone sodium phosphate media (HC; Merke, Sharpe & Dohme, West Point, Pa.) HC was readily soluble in RPMI 1640 culture medium and used in final concentrations of 10, 5, and 1 μg/ml during the primary culture phase of the experiment. Dilutions of hydrocortisone were made with the standard control medium.

Secondary Phase Cultures

All secondary cultures were performed in control RPMI 1640 media without CY-A, HC, or DMSO. Fresh stimulator cells were irradiated with 2500 rads and designated as B_s (original reference cell), or C_s or D_s (third-party stimulators). The responding primed lymphocytes ($A'B_s$), 5×10^5 cells/ml were cultured with 10^6 stimulator cells/ml in a microculture system. All cultures were done in quadruplicate. Equal volumes of responder cells and stimulator cells (total 0.2 ml) were added to microtiter wells and incubated from 0 to 5 days. At the end of these predetermined periods, each microculture was pulse-labeled with 1.0 μCi tritiated thymidine (^{3}H-Tdr; Schwartz/Mann, Inc., Orangeburg, N.J.; specific activity, 6 Ci/m*M*) and allowed to incubate an additional 12–18 hr. Cells were harvested onto glass-fiber filter paper with a MASH unit and ^{3}H-Tdr incorporation subsequently counted in a Packard liquid scintillation counter.

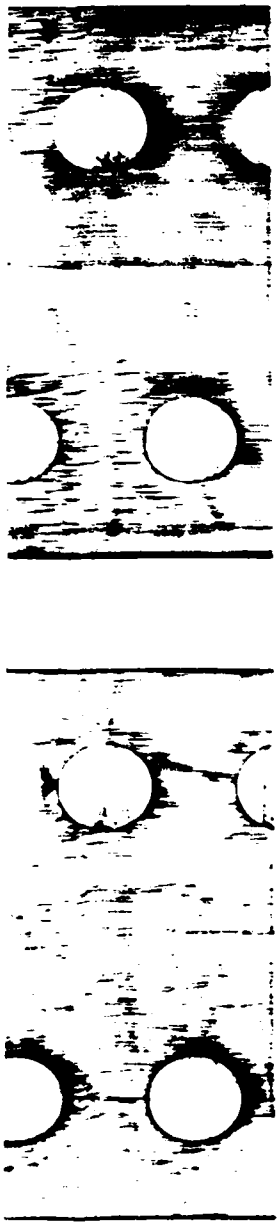
RESULTS

Primed Lymphocyte Recovery Rates

Lymphocyte viability was determined by trypan blue exclusion. The yield of primed lymphocytes compared to the original responder population (16×10^6) is noted in Table 1. There was a 92% recovery of viable primed lymphocytes in the control media. Unlike controls, all DMSO-treated cultures (with or without CY-A) had an extremely poor recovery, ranging from 16% to 27%. Hydrocortisone-treated cells yielded intermediate recovery of viable lymphocytes between 48% and 60%.

Table 1. Lymphocyte Recovery Rates From Priming Culture Media

	Total Cells Harvested × 10^6	Viability (%)	Viability (Cells × 10^6)	Percent Yield (Viable Cells)
Control	16.3	90%	14.7	92%
DMSO 1% v/v	3.6	86%	3.1	19%
Cyclosporin A				
0.1 μg/ml	4.3	62%	2.6	16%
0.5 μg/ml	6.1	70%	4.3	27%
1.0 μg/ml	5.5	54%	3.0	19%
Hydrocortisone				
1.0 μg/ml	11.4	84%	9.6	60%
5.0 μg/ml	12.7	69%	8.8	55%
10.0 μg/ml	9.6	80%	7.7	48%



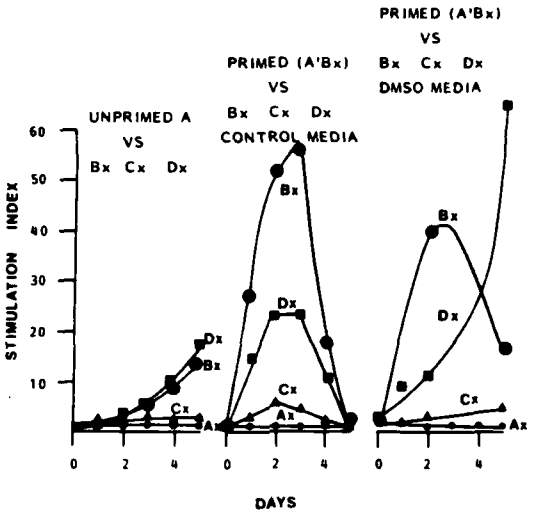


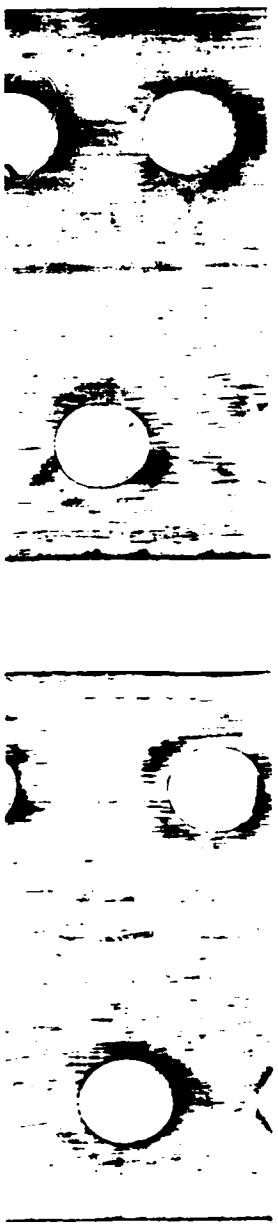
Fig. 1. Effect of standard control and DMSO control primed cells on B_x, C_x, and D_x stimulators. B_x is the original reference stimulator cell. Also noted is the response of unprimed A cells to B_x, C_x, and D_x. The stimulation index was calculated by dividing a mean experimental response by a mean control autologous response for each appropriate media and time period.

Primed Cell Response in Secondary Cultures

Controlled media. Figure 1 illustrates the response of control primed cells $(A'B_x)_{cont}$, DMSO-primed cells $(A'B_x)_{DMSO}$, and unprimed cells (A) to individual stimulator populations (B_x, C_x, D_x). There were temporal and quantitative differences in the respective responses. The $[(A'B_x)_{cont} + B_x]$ response was as expected on day 2 with a stimulation index (SI) of 58 compared to an SI of 1.22 for the unprimed $A + B_x$ culture. $(A'B_x)_{DMSO}$ cells also demonstrated an augmented response on day 2 (SI, 40) to reference cell B_x, while the peak response to third-party control cells was much lower. The response of $[(A'B_x)_{DMSO} + B_x]$ was 45,000 ± 1810 cpm compared to 156,000 ± 5479 cpm of $[(A'B_x)_{cont} + B_x]$ on day 2. This lower response is not surprising since DMSO is known to inhibit DNA synthesis. Thus, it was necessary to compare CY-A-primed lymphocytes to the DMSO control media-primed lymphocytes, while HC-primed lymphocytes were compared to the standard control media-primed lymphocytes.

Cyclosporin A media. These experiments were performed to see if lymphocytes in a primary mixed lymphocyte reaction (MLR) with various concentrations of CY-A would have the ability to develop memory cells, i.e., primed lymphocytes. This was tested by comparing the secondary response of these cells $(A'B_x)_{CY\text{-}A}$ with B_x, C_x, or D_x, to the secondary response of $(A'B_x)_{DMSO}$ to those same stimulators. The $(A'B_x)_{CY\text{-}A}$ cells did not respond to stimulator cells, neither reference (B_x) nor third-party cells (C_x or D_x) on days 1, 2, or 3. (Fig. 2). Despite this early unresponsiveness, there was definite activity of $(A'B_x)_{CY\text{-}A}$ by day 5. This response was apparent in all CY-A concentrations and was not dose dependent in the range tested. $(A'B_x)_{DMSO}$ cells did exhibit an early secondary primed response to reference stimulator B_x but not to C_x, or D_x. The mean response of $(A'B_x)_{CY\text{-}A}$ to B_x on day 5 was equal in magnitude (48,508 ± 4366 cpm) to the early primed response (45,302 ± 1810 cpm) seen with $(A'B_x)_{DMSO}$ on day 2. Also the $(A'B_x)_{CY\text{-}A}$ response was 4.5 times greater on day 5 than the unprimed cells ($A + B_x$) noted in the routine MLR. This indicates that $(A'B_x)_{CY\text{-}A}$ maintained the ability to respond de novo in secondary cultures as long as CY-A was not present in the medium.

Hydrocortisone media. The effect of hydrocortisone on lymphocyte priming is noted in Fig. 3. $(A'B_x)_{HC}$ responsitivity to B_x,



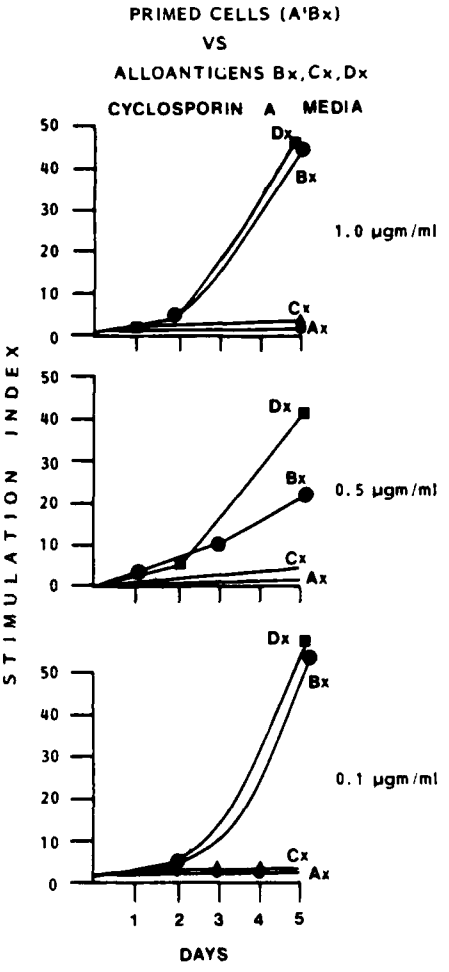


Fig. 2. Response of cyclosporin-A-primed cells in secondary culture to original stimulator B_x or third-party stimulators C_x, D_x. Concentrations of CY-A were used only during the priming phase and not in secondary cultures.

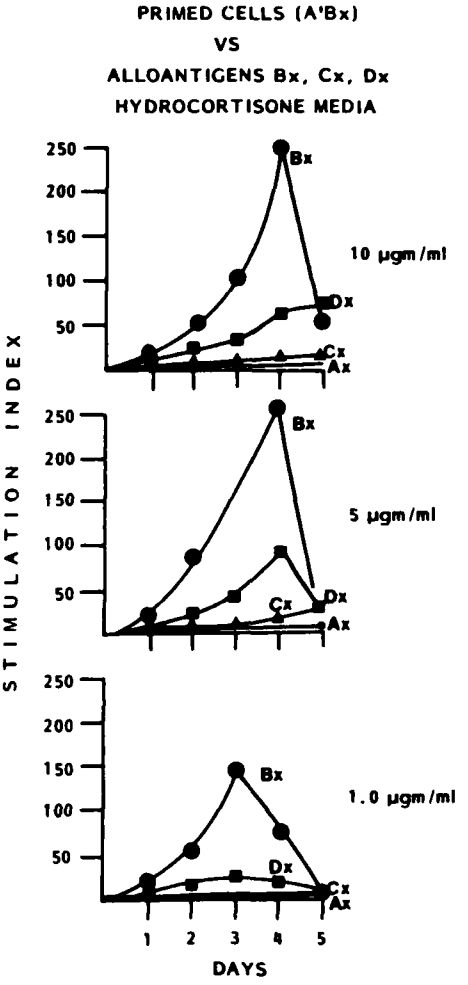
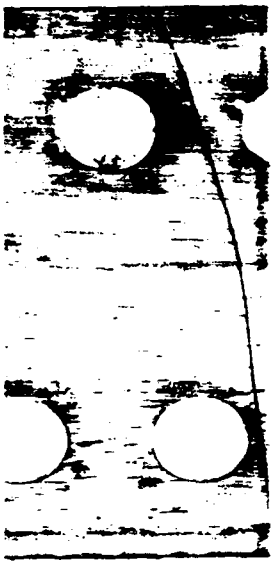
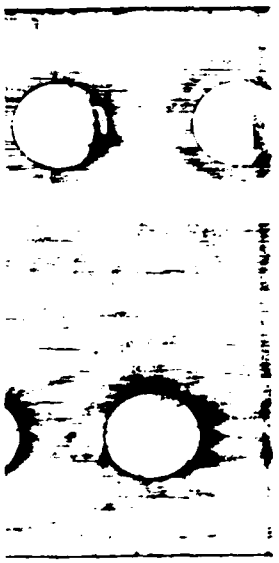


Fig. 3. Response of hydrocortisone-primed cells in secondary culture to original stimulator B_x or third-party stimulators C_x, D_x. Concentrations of HC were used only during the priming phase and not in secondary cultures.

C_x, D_x was delayed by 24 hr, compared to $(A'B_x)_{cont}$. The peak response to reference stimulator B_x was seen on day 3 instead of day 2. There was no inhibition of the response with $(A'B_x)_{HC}$, in fact, the response was augmented. The memory of $(A'B_x)_{HC}$ was unaffected since the stimulation of these cells to reference cell B_x was earlier and more vigorous than to C_x or D_x stimulators.

As noted in Fig. 4. $(A'B_x)_{CY\text{-}A}$ is inhibited early in the culture system, but this inhibition is lost by day 5. The inhibition is most marked (96%) against reference cell B_x, but $(A'B_x)_{CY\text{-}A}$ also responded less to C_x and D_x over the same time period. $(A'B_x)_{HC}$ cells were found to show no preferential inhibition to reference cell B_x or third-party stimulators C_x or D_x. Unlike CY-A, therefore, HC did not interfere with lymphocyte priming.





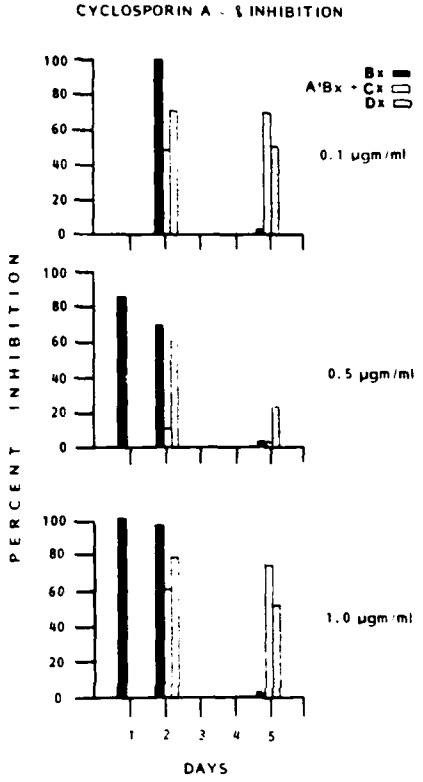


Fig. 4. Inhibition of cyclosporin-A-primed cell responses to specific stimulators in secondary cultures. Inhibition was calculated as:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{cpm Exp.} - \text{cpm Media}}{\text{cpm Control} - \text{cpm Media}}\right) \cdot 100$$

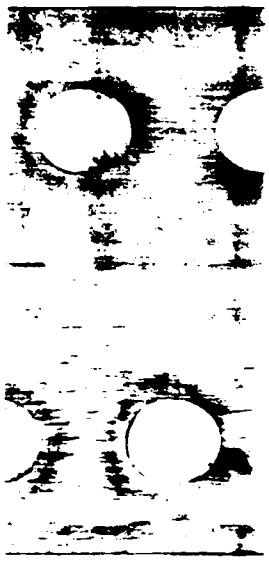
where Exp. = mean response of $(A'B_x)_{CY\text{-}A}$ or $(A'B_x)_{HC}$ to a stimulator cell; Control = mean response of $(A'B_x)_{DMSO}$ or $(A'B_x)_{con}$ to a stimulator cell; Media = spontaneous ^{3}H-Tdr uptake.

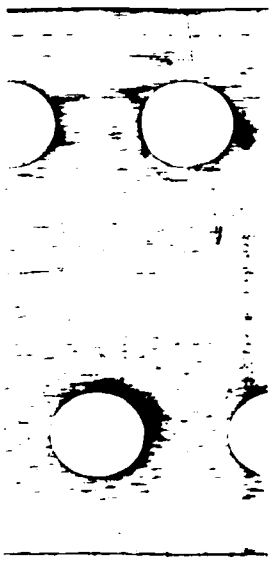
DISCUSSION

We chose to examine the mechanism of CY-A in a primed lymphocyte culture model. It is known that lymphoid cells from two unrelated individuals, when mixed together, will undergo blastogeneses.[12,13] The recognition of antigenic determinants on the stimulator population will lead to a generation of lymphoid cells that have specific memory for those determinants.[14-16] These memory cells, when cultured in the secondary phase mixed lymphocyte reaction (MLR) with their primary reference stimulator cell, will respond rapidly and vigorously usually within the first 48–72 hr of culture. Unprimed cells, however, will respond in a more routine manner, generally developing a maximum response at day 5 rather than day 2 or 3.[16]

If CY-A inhibits only blastogenic cells in MLR, we hypothesized that it might inhibit the formation or the response of primed (memory) cells. We also reasoned that other lymphocytes not responding to the reference stimulator in the priming culture would respond in a routine fashion to third-party alloantigens in a secondary culture. The results have confirmed our hypothesis since $(A'B_x)_{CY\text{-}A}$ cells did not respond to reference stimulator B_x in a primed fashion in the secondary culture on days 1 or 2. HC had no such effect on the primed cells, since $(A'B_x)_{HC}$ responded in a primed manner to B_x the same as controls did. Also, the primed response noted by $(A'B_x)_{DMSO}$ to B_x on day 2 makes it certain that the solvent DMSO is not responsible for the failure of $(A'B_x)_{CY\text{-}A}$ to develop a primed response. In addition, $(A'B_x)_{CY\text{-}A}$ responded at day 5 to the original reference cell and third-party stimulators, indicating the CY-A effect was gone at this time. A disturbing finding in the experiment was the lack of a dose response to CY-A, since other in vitro studies with this drug have shown dose dependence.

It might be argued that the data are a consequence of inadequate washing of CY-A during the transfer of cells from priming to secondary cultures. If this were the case and CY-A was bound to the cellular membranes or present in the cytoplasm, we would expect continuous suppression of these cells from day 0 through 5. Leoni and colleagues[11] reported that ^{125}I-CY-A was rapidly taken up by lymphocytes, whether resting or stimulated. However, complete elimination of the drug was obtained in as little time as 6–7 hr, if the cells were cultured in a CY-A-free medium. They also reported that if CY-A-treated cells were first washed and then resuspended in medium lacking the drug, but containing PHA, they would show considerable mito-





genic recovery within the first 48 72 hr. Furthermore, data from our laboratory, (unpublished) have shown that MLR is suppressed with CY-A at a 50% inhibition (ID_{50}) of 0.5 μg/ml. White and colleagues[10] have found similar results. They reported that porcine-primed cells were inhibited with CY-A at an ID_{50} of 1.0 μg/ml in the secondary phase cultures. Since our initial concentrations of CY-A ranged between 0.1 and 1.0 μg/ml, it is highly unlikely, after the multiple washings, that enough CY-A was carried over to effect the secondary phase cultures significantly.

Although the mechanisms of immunosuppression remain unclear, several in vitro and in vivo studies have now shown that this agent inhibits thymus-dependent humoral and T-cell immune responses.[2,7] In addition, T-cell responsiveness to PHA and Con-A mitogenesis is inhibited in a dose-dependent manner with an ID_{50} of 0.5 μg/ml and 0.1 μg/ml, respectively (unpublished data). Clearly, the agent suppresses only blastogenic cells and has little effect on unstimulated cells.

From the in vivo and in vitro observations, three theories regarding mechanisms have been proposed. The first is a speculation that CY-A completely eliminates clones of cells and thus creates tolerance in certain species.[3,9] Our results would tend to disprove this concept, since we found that $(A'B_1)_{CY\ A}$ cells were able to respond to both original reference stimulator and third-party stimulators by the fifth day in secondary culture. This suggests that CY-A must be continually present to inhibit cellular responses to alloantigenic stimulation, and total elimination of the cells did not occur. Second, Gordon and Singer[18] infer that the mechanism of CY-A may be an inhibition to the T "helper" cell population. Our experimental model was unable to define which subpopulation of T cells was inhibited by CY-A. However, the continued presence of the drug was necessary to obtain the results. Finally, an interesting concept regarding the mechanism of CY-A has been raised by Tutschka and colleagues.[4] They speculate that the tolerance inferred by CY-A in a rat bone marrow model may be the result of an accelerated appearance of a T-suppressor cell population. Our results would not support this concept, since one would have expected any nonspecific suppressor cell activity to be operative throughout the secondary phase cultures. Also, the significant loss of cells after 10 days of primed cultures makes it unlikely that the mechanism of this drug is activation or stimulation.

In conclusion, the data provide evidence that lymphocytes, primed in vitro against specific alloantigens in the presence of CY-A, lost their ability to respond in an accelerated or primed manner to those same alloantigens in a secondary culture. This was not true of cells primed in vitro with hydrocortisone. However, CY-A cells were able to respond to original alloantigens by the fifth day in a secondary culture. These data suggest that the constant presence of CY-A is necessary to exhibit its antilymphocytic activity.

REFERENCES

1. Calne RY. IRCS Med Sci 6:447, 1978
2. Borel JF, Feurer C, Gubler HU, et al. Agents Actions 6:468, 1968
3. Green CJ, Allison AC. Lancet 1:1182, 1978
4. Tutschka PJ, Beschorner WE, Allison AC, et al. Nature 280:148, 1979
5. Powles RL, Clink H, Sloane J, et al. Lancet 2:1327, 1978
6. Calne RY, Thiru S, McMaster P, et al. Lancet 2:1323, 1978
7. Jamieson SW, Burton NA, Bieber CP, et al. Lancet 1:545, 1979
8. Green CJ, Allison AC, Precious S. Lancet 2:123, 1979
9. Dunn DC, White DJG, Wade J. IRCS Med Sci 6:464, 1978
10. White DJG, Plumb AM, Powles G, et al. Transplantation 27:55, 1979
11. Leoni P, Garcia RC, Allison AC, et al. J Clin Lab Immunol 1:67, 1978
12. Bain B, Vas MR, Lowenstein L. Blood 23:108, 1964
13. Bach FH, Hirshhorn K. Science 143:813, 1964
14. Sheehy MJ, Sondel PM, Bach ML, et al. Science 188:1308, 1975
15. Hartzman RJ. Tissue Antigens 13:203, 1979
16. Singal DP. Transplant Proc 9:709, 1977
17. Borel JF, Wiesinger D, Gubler HU. Eur J Rheum 237, 1978
18. Gordon MY, Singer JW. Nature 279:433, 1979

0041-1337/80/3006-0404$02.00/0
TRANSPLANTATION

Vol. 30, No. 6
Printed in U.S.A.

IN VITRO EFFECTS OF CYCLOSPORIN A ON LYMPHOCYTE SUBPOPULATIONS

1. SUPPRESSOR CELL SPARING BY CYCLOSPORIN A[1]

STEPHEN B. LEAPMAN, RONALD S. FILO, EDWIN J. SMITH, AND PATRICIA G. SMITH

Departments of Surgery (Transplantation) and Medicine (Nephrology), Indiana University Medical Center, Indianapolis, Indiana 46202

SUMMARY

The fungal metabolite, cyclosporin A, is a potent immunosuppressive compound. Experiments were performed in vitro with both human and nonhuman primate peripheral blood lymphocytes to study the effect of this agent on suppressor cell activity. Cyclosporin A did not affect the generation or function of concanavalin A-induced suppressor lymphocytes as measured by their ability to suppress thymidine uptake of lymphocytes in secondary cultures. No evidence of suppressor cell induction was noted by incubation of lymphocytes with only cyclosporin A. We conclude that, although cyclosporin A does not generate or induce suppressor cell lymphocytes, it does spare them, while inhibiting other subpopulations. This effect may create an imbalance in the immune system which results in profound suppression.

Those cells responsible for the induction of the immune system are the lymphocytes. It is now established that these immune responses are modulated and regulated by subsets of lymphocytes, specifically, the thymus-derived (T) cells which can either suppress or augment humoral or cellular immune reactions (*1–3*). In addition to the autoregulatory mechanisms, certain pharmacological and biological agents can profoundly effect the activity of these lymphocytes, creating immunosuppressive or immunoenhancing milieus. Such an immunosuppressive agent is cyclosporin A (CS-A), an antifungal, cyclic endecapeptide with a unique amino acid (*4*).

This compound has been reported to be antilymphocytic (*5–7*), but its effect on specific subpopulations of lymphocytes has received little attention. Our laboratory has been interested in this compound and in vitro assays have been used to probe its immunosuppressive mechanisms. We recently reported the effect of CS-A on memory cell function and its ability to suppress primed lymphocytes against specific alloantigens (*8*). Gordon and Singer (*9*) have inferred that CS-A may inhibit helper T cell populations while Tutschka *et al.* (*10*) suggest that tolerance induced by CS-A in a rat bone marrow model may be the result of an accelerated appearance of the T suppressor cell population. To better understand the influence of this agent on suppressor cell populations, in vitro experiments were designed to answer the following questions: (1) Does CS-A inhibit the generation of concanavalin A (Con A)-induced suppressor cells? (2) Can CS-A inhibit the function of these suppressor cells? (3) Will CS-A alone stimulate the formation of suppressor cells? (4) is the effect of CS-A on Con A generated suppressor cells dose dependent? The results of these experiments form the basis of this report.

[1]This work was supported by ONR Grant Contract N000014-78-C-0412. Presented at the Sixth Annual Meeting of the American Society of Transplant Surgeons, Chicago, Illinois, May 29 to 31, 1980.

MATERIALS AND METHODS

Mononuclear Cell Preparation

Peripheral blood was obtained by venipuncture from healthy human volunteers. Mononuclear cells were isolated by Ficoll-Hypaque discontinuous gradients (specific activity, 1.078). All cells were washed with RPMI 1640, counted, and cultured as noted below. In some experiments, specifically noted, peripheral blood lymphocytes (PBL) were obtained from healthy stumptailed macaques (*Macaca speciosa*) and processed the same as human cells.

Preparation of Suppressor Cells

Primary culture. Medium used in these experiments was RPMI 1640 with 10% pooled human or monkey plasma supplemented with HEPES (25 mM), bicarbonate (20 g/liter), L-glutamine (20 mg/liter), and gentamycin (80 mg/liter). Con A (10 to 25 μg/ml; Sigma, St. Louis, Missouri) alone or in combination with CS-A (0.1 μg/ml; Sandoz, Ltd., Basel, Switzerland) was added to the above culture medium. Control cells (nonactivated) were incubated in medium only. Since CS-A is insoluble in water, it was dissolved in absolute ethyl alcohol to a final concentration of 0.2%. This concentration of alcohol was present in all culture media and did not effect lymphocyte responsiveness. In one experiment using lymphocytes from *M. speciosa*, only CS-A (0.1 μg/ml) was added at the beginning to the primary culture to determine if this agent would stimulate generation of suppressor cells. In a second set of experiments macaque lymphocytes were stimulated with Con A and various dose dilutions of CS-A (0.01 to 1.0 μg/ml). These mitogen-stimulated lymphocytes (suppressor cells) were cultured in 25-cm^2 flasks at 1×10^6 cells/ml. Incubation was carried out in a 5% CO_2 humidified atmosphere for 72 hr at 37 C. At the end of 3 days, these cells were harvested, washed two times with 75 mM α-methyl-D-mannoside, and twice with RPMI 1640. All cells were counted and viability was assessed by trypan blue exclusion. Functional viability of these primary cultured cells was determined by plating aliquots of control, Con A, or CS-A + Con A cell cultures into microtiter wells. These aliquots were labeled with 1.0 μc of tritiated thymidine [^{3}H]TdR (Schwarz/Mann, Inc., Orangeburg, New York; specific activity, 6 c/mM) for an additional 12 to 18 hr. Cells were harvested onto glass filter fiber paper with a MASH unit and [^{3}H]TdR incorporation was subsequently counted in a Packard liquid scintillation counter. These day 0 responses were necessary to examine the inhibitory effect of CS-A on the Con A-treated cells. The remaining primary cultured cells were irradiated with 2,500 to 4,000 rad and subsequently placed in secondary cultures to assay for suppresion.

Secondary cultures. Fresh cells from the human volunteers

or the macaques were obtained and separated as described above. These cells were then cultured in microtiter wells (1×10^5 well) with phytohemagglutinin (PHA) (1%), Con A (25 μg/ml) or pokeweed (0.5 μg/ml) mitogens for 3 days. For certain experiments cells were stimulated with irradiated (2,500 R) alloantigeneic or autologous lymphocytes as a mixed lymphocyte culture (MLC) for 5 days.

Assay for suppressor activity. Each population from the primary cultures was assayed for suppressor activity in the secondary cultures described above. These putative suppressors were added to the secondary cultures at 1×10^5 cells/well. All responding cells in the secondary culture system were autologous with the suppressor cells. At the end of the respective culture period, the cells were pulse labeled with [^{3}H]TdR 12 to 18 hr, harvested, and counted in a Packard liquid scintillation counter.

TABLE 1. Human PBL recovery rates and [^{3}H]TdR incorporation after 72 hr of primary culture

Cells incubated with	% cells recovered from original culture	% viable cells*	[^{3}H]TdR incorporation	
			cpm ± SEM	% suppression†
Nonactivated	81	92	89 ± 5	
Con A	152	78	49,625 ± 474	86
Con A + CS-A	135	87	7,171 ± 216	
Nonactivated	94	91	256 ± 14	
Con A	133	89	33,619 ± 1,185	56
Con A + CS-A	89	93	14,861 ± 286	
Nonactivated	61	90	120 ± 9	
Con A	72	86	78,448 ± 1,790	65
Con A + CS-A	92	87	27,676 ± 839	
Nonactivated	119	94	62 ± 10	
Con A	104	94	40,028 ± 445	56
Con A + CS-A	93	89	17,576 ± 756	
Nonactivated	98	95	271 ± 40	
Con A	180	96	16,569 ± 184	76
Con A + CS-A	107	99	3,932 ± 239	
Nonactivated	55	87	482 ± 10	
Con A	103	92	34,950 ± 867	60
Con A + CS-A	47	92	14,047 ± 788	
Nonactivated	81	91	12,183 ± 268	
Con A	144	94	60,625 ± 3,971	72
Con A + CS-A	77	89	16,725 ± 466	
			x̄ ± SEM = 67 ± 11	

* Determined by trypan blue exclusion.

† Suppression is effect of Con A + CS-A (0.1 μg/ml) compared to Con A-activated cells.

Data and statistics. Data from mitogen-stimulated experiments are expressed as mean cpm ± SE of quadruplicate cultures. Percentage of suppression was calculated as

$$\% \text{ suppression} = 1 - (\text{cpm NA}/\text{cpm Exp}) \times 100$$

where cpm Exp = cpm of secondary cultures with Con A or Con A + CS-A cells and cpm NA = cpm of secondary cultures with cultured but nonactivated control cells.

Alloantigen stimulated responses are expressed as stimulation indices (SIs). The SI is a ratio of the allogeneic stimulated response divided by the autologous stimulated response. The percentage of suppression in these experiments were done as follows:

$$\% \text{ suppression} = 1 - (\text{SI experimental}/\text{SI control}) \times 100$$

where SI experimental = allogeneic responses with Con A or Con A + CS-A added cells and SI control = allogeneic response with cultured but nonactivated control cells present in the secondary culture. The significance of the suppression as described above was tested by Student's one-tailed *t*-test or an analysis of variance (F).

RESULTS

Effects of Con A or CS-A on Primary Cultured Cells

Table 1 shows the recovery rates and proliferative responses of human lymphocytes in primary cultures as determined by viable cell counts. After 72 hr there was a mean recovery rate of 126% in Con A-cultured cells compared to the original number of cells placed in culture. The combination of CS-A and Con A resulted in a 91% recovery rate compared to an 84% recovery rate of the cells cultured but not activated. A significant suppression, 67 ± 11%, in [^{3}H]TdR uptake was noted in those cultures containing Con A + CS-A when compared to Con A cultures. This inhibitory response is similar to previous experiments done in this laboratory where the dose of 0.18 μg of CS-A per ml was necessary to create a 50% inhibitory response (ID_{50}). We also observed that Con A-stimulated cultures were nearly 100% blasts while Con A + CS-A cultures had less than 10% blasts.

TABLE 2. Suppressor activities of Con A or Con A + CS-A-stimulated human lymphocytes on the mitogenic responses of autologous lymphocytes

Experiment	Addition of nonactivated, Con A, or Con A + CS-A suppressors	[^{3}H]TdR incorporation stimulated with					
		PHA		Con A		PWM	
		cpm ± SEM	% suppression*	cpm ± SEM	% suppression	cpm ± SEM	% suppression
1	Nonactivated	222,311 ± 10,379		189,453 ± 5,112		65,301 ± 2,427	
	Con A	175,870 ± 9,533	21	167,163 ± 8,310	12	81,872 ± 7,038	0
	Con A + CS-A	173,532 ± 8,437	22	174,512 ± 4,135	8	67,206 ± 3,835	0
2	Nonactivated	147,897 ± 10,055		149,194 ± 2,666		85,842 ± 3,715	
	Con A	130,588 ± 11,086	12	123,151 ± 4,207	17	58,280 ± 1,465	32
	Con A + CS-A	132,045 ± 19,911	11	112,910 ± 4,921	24	63,176 ± 3,087	26
3	Nonactivated	235,687 ± 6,858		212,430 ± 4,229		108,800 ± 2,407	
	Con A	194,217 ± 3,037	18	171,818 ± 2,158	19	90,483 ± 2,979	17
	Con A + CS-A	272,145 ± 8,979		181,474 ± 5,071	15	85,874 ± 2,675	21
4	Nonactivated	278,225 ± 1,321		125,517 ± 3,266		138,597 ± 2,281	
	Con A	262,885 ± 4,156	6	121,269 ± 4,902	4	121,057 ± 2,802	13
	Con A + CS-A	268,280 ± 8,109	4	111,677 ± 6,598	11	133,588 ± 2,395	4
x̄ ± SEM	Con A		14.2 ± 3		13.0 ± 3		15.5 ± 6
	Con A + CS-A		9.2 ± 4		14.5 ± 3		12.7 ± 6

* Con A and Con A + CS-A suppression is significant by the one-tailed Student's *t*-test ($P < 0.05$) when compared to responses of nonactivated cultured cells. However, Con A compared to Con A + CS-A suppression do not differ from each other when tested by a one-tailed Student's *t*-test ($P > 0.05$).

Effects of NA, Con A-, or Con A + CS-A-treated Cells on the Proliferative Responses of Autologous Cells

Mitogenic responses. The suppression conferred on PBL mitogen-induced proliferation is shown in Table 2. The suppression demonstrated by both Con A and Con A + CS-A-treated cells was similar for all three stimulatory mitogens. The degree of suppression comparing Con A to Con A + CS-A suppressor cells was not significantly different in these experiments. It is important to note that the proliferative responses in these secondary cultures are from the responding autologous cells and not from the added suppressor cells since they had been irradiated prior to addition to the secondary culture. Also, there was no carryover of Con A or CS-A into the secondary cultures since uptake of [^{3}H]TdR of nonactivated, Con A, or Con A + CS-A suppressor cells were not different in unstimulated cultures containing these cells only.

Allogeneic responses. Suppressor cells were added to mixed lymphocyte reactions. The results in Table 3 demonstrate significant suppression by both Con A and Con A + CS-A cells. There was a 64% mean suppression induced by the Con A cells, compared to the 83% noted by the Con A + CS-A suppressors. These responses are statistically different as determined by an analysis of variance, $F = 7.58$ ($P < 0.05$). Suppression was more pronounced with the more specific allogeneic stimulation than with the more potent but less specific mitogens.

CS-A Dose Responses in Primary Cultures (M. speciosa)

These experiments were performed with PBLs from *M. speciosa*. We have previously shown that these cells respond to mitogens, alloantigens, and CS-A in vitro similar to human cells. A dose response curve through 2 log increments was performed. Figure 1 illustrates that CS-A in doses from 0.01 to 1.0 μg/ml had no effect on the generation of Con A suppressor cells and that these cells were able to suppress an allogeneic response in MLR by 50%. Doses higher than 1.0 μg/ml were not done, however, and it is possible that an increased concentration could decrease Con A-induced suppressor cell activity.

TABLE 3 Suppression of allogeneic responses of normal human lymphocytes by Con A or CS-A + Con A pretreated autologous cells

Responding cell	Stimulator	Suppressor cell	cpm ± SEM	SI[a]	% suppression[b]
A	Ax	A_{NA}[c]	180 ± 11	1.0	—
	Bx	A_{NA}	23,002 ± 2,946	127.8	—
	Cx	A_{NA}	36,718 ± 2,679	203.0	—
A	Ax	A Con A	156 ± 46	1.0	—
	Bx	A Con A	6,302 ± 994	40.4	68
	Cx	A Con A	10,244 ± 493	65.7	68
A	Ax	A Con A + CS-A	301 ± 88	1.0	—
	Bx	A Con A + CS-A	11,240 ± 615	37.3	71
	Cx	A Con A + CS-A	17,620 ± 596	58.5	71
B	Ax	B_{NA}	43,914 ± 5,924	106.1	—
	Bx	B_{NA}	414 ± 33	1.0	—
	Cx	B_{NA}	60,111 ± 6,309	145.2	—
B	Ax	B Con A	20,628 ± 2,478	21.4	80
	Bx	B Con A	964 ± 235	1.0	—
	Cx	B Con A	23,863 ± 1,528	24.8	83
B	Ax	B Con A + CS-A	36,210 ± 3,404	4.5	96
	Bx	B Con A + CS-A	7,980 ± 1,710	1.0	—
	Cx	B Con A + CS-A	46,985 ± 4,088	5.8	96
C	Ax	C_{NA}	116,449 ± 7,922	101.4	—
	Bx	C_{NA}	101,253 ± 2,937	88.2	—
	Cx	C_{NA}	1,148 ± 518	1.0	—
C	Ax	C Con A	93,134 ± 4,553	55.0	46
	Bx	C Con A	88,186 ± 2,920	52.1	41
	Cx	C Con A	1,692 ± 410	1.0	—
C	Ax	C Con A + CS-A	31,619 ± 1,356	26.2	74
	Bx	C Con A + CS-A	29,176 ± 1,210	24.2	73
	Cx	C Con A + CS-A	1,205 ± 115	1.0	—
D	Ax	D_{NA}	16,321 ± 1,020	53.3	—
	Bx	D_{NA}	36,752 ± 3,826	120.1	—
	Dx	D_{NA}	306 ± 37	1.0	—
D	Ax	D Con A	5,643 ± 1,128	15.8	71
	Bx	D Con A	17,081 ± 3,813	48.0	60
	Dx	D Con A	356 ± 56	1.0	—
D	Ax	D Con A + CS-A	7,480 ± 1,012	3.2	94
	Bx	D Con A + CS-A	26,382 ± 1,441	11.4	91
	Dx	D Con A + CS-A	2,318 ± 403	1.0	—
$\bar{x}$ ± SEM Suppression %[d]		Con A	64.6 ± 5	Con A + CS-A	83.2 ± 4

[a] $SI = \frac{\text{cpm allogeneic response}}{\text{cpm autologous response}}$

[b] $\%\ \text{suppression} = 1 - \left(\frac{\text{SI experimental suppressors}}{\text{SI NA suppressors}}\right) \times 100$

[c] NA are nonactivated but cultured cells in primary culture.

[d] Percentage of suppression is significantly different between Con A and Con A CS-A as judged by an analysis of variance ($F = 7.58$).

Generation of Suppressor Cells by CS-A Alone in Primary Cultures

Since it was feasible that CS-A alone could generate suppressor cells, this series of experiments was performed. Table 4 illustrates that in general CS-A-treated primary cells do not show suppressor activity. One animal, however, did develop suppressor cell activity against mitogen responses which was equal to that induced by Con A suppressors. None of the other animals tested, however, were able to suppress significantly an allogeneic response and we conclude that these experiments demonstrate the inability of CS-A to generate suppressor cells. These experiments also indicate that there was no carryover of CS-A from primary cultures, since the secondary culture responses with the addition of these cells were at least equal to or greater than the addition of nonactivated but cultured cells to a secondary culture.

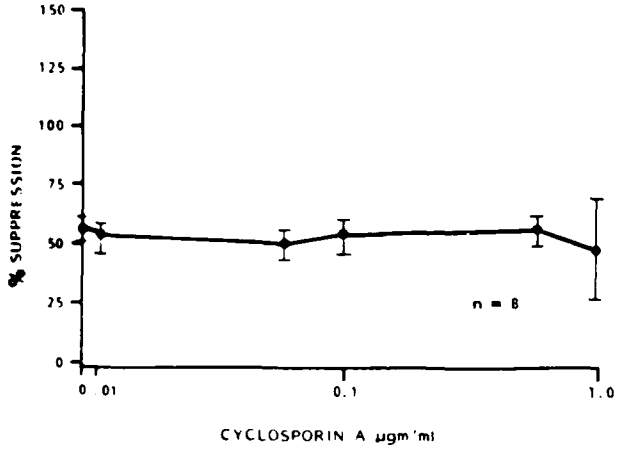


FIGURE 1 Dose response curve of CS-A (means ± SE) added to a constant concentration of 25 µg of Con A per ml in primary culture. There was no difference in percentage of suppression between 0.01 and 1.0 µg of CS-A per ml when these cells were tested in secondary cultures for suppressor activity (*M. speciosa*).

DISCUSSION

Although the mechanism of immunosuppression by CS-A remains unclear, several in vivo and in vitro studies in multiple species have now shown that this compound inhibits thymus-dependent (T cell) humoral and cell-mediated immune responses (*4–6, 11, 12*). Its actions may be augmented in vivo, however, by the sparing of suppressor cells, thereby enhancing the relative number of these cells as a result of a diminished T helper cell subpopulation.

Shou et al. (*13*) reported on a population of cells present in the peripheral blood of normal humans that could be induced by Con A to manifest suppressor cell function. These cells, whether autologous or allogeneic, inhibited blast transformation and [^{3}H]TdR incorporation by untreated lymphocytes in both mixed lymphocyte reactions and mitogenic or antigenic stimulated situations. It was further demonstrated by these investigators that the effect occurred without evidence of cytotoxicity. Others (*14*) have reported, however, that cytotoxic effector cells can be activated by Con A incubation under very specific in vitro conditions. Sakane and Green (*15*) concluded that those cells induced by Con A to suppress cell to cell reactions were thumus (T) cell dependent and that Con A-stimulated B cells did not exhibit suppressor activity. Further separation of these cells by discontinuous bovine serum albumin gradients indicated that high but not low density T cells produced marked suppression, yet this fraction of cells incorporated little thymidine when exposed to Con A. This finding is in agreement with experiments (*16*) done with murine cells where suppressors (Ly-2,3) were found in a blast cell fraction and their induction was not inhibited by prior treatment with irradiation or mitogens (*16*).

This study examined the in vitro effects of CS-A on a sup-

TABLE 4 Effect of CS-A-pretreated cells in secondary cultures with autologous responders stimulated by either mitogens or alloantigens (*M. speciosa*)

A *Mitogens*

Experiment	CS-A pretreatment from primary culture	[^{3}H]TdR incorporation in response to mitogens stimulated with					
		PHA		Con A		PWM	
		cpm ± SEM	% suppression	cpm ± SEM	% suppression	cpm ± SEM	% suppression
1	–	42,542 ± 924		29,037 ± 657		126,872 ± 7,601	
	+	35,644 ± 943	16*	23,401 ± 761	19	99,984 ± 3,038	21
2	–	53,307 ± 2,316		43,680 ± 1,748		40,988 ± 1,302	
	+	50,680 ± 543	5	44,785 ± 1,801	0	43,826 ± 1,216	0
3	–	131,426 ± 2,121		175,477 ± 2,755		157,243 ± 5,344	
	+	145,470 ± 1,922	0	364,300 ± 462	0	176,205 ± 8,415	0

B *Alloantigens*

Experiment	CS-A pretreatment from primary culture	Responder	Response to alloantigens when stimulated by irradiated lymphocytes								
			Bx			Cx			Dx		
			cpm ± SEM	SI†	% suppression	cpm ± SEM	SI	% suppression	cpm ± SEM	SI	% suppression
4	–	B	2,491 ± 228	1.0		5,917 ± 816	2.38		6,775 ± 947	2.72	
	+		1,770 ± 185	1.0	0	7,188 ± 532	4.06	0	13,462 ± 1,901	7.61	0
5	–	C	18,528 ± 2,903	7.29		2,540 ± 465	1.0		21,767 ± 2,543	8.57	
	+		24,185 ± 1,873	10.66	0	2,268 ± 223	1.0	0	30,948 ± 4,459	13.50	0
6	–	D	26,784 ± 1,727	8.53		22,454 ± 1,330	7.15		3,139 ± 355	1.0	0
	+		22,245 ± 2,043	6.02	29*	19,923 ± 1,638	5.39	25	3,696 ± 661	1.0	

* Percentage of suppression for experiment 1 is significant as judged by Student's one-tailed *t*-test ($P < 0.05$). Suppression in experiment 6 is not significant

† SI, calculations described in Materials and Methods.

pressor cell population in both human and nonhuman primate cultured cells. The results indicate that Con A-induced suppressor cells are uneffected by coculture with CS-A despite a marked inhibition (67% ± 11%) of [^{3}H]TdR uptake in those CS-A + Con A cultured primary cells. The addition of CS-A to the primary culture also reduces the number of blast cells present at the end of the incubation period. The remaining cells still suppressed mitogenic responses in secondary cultures equal to those suppressor cells induced by Con A alone and significantly more suppression was noted by these cells in allogeneic responses. It would seem reasonable to conclude that CS-A had no detrimental effect on the generation of suppressor cells and enhanced their activity in suppressing the alloantigeneic responses. However, kinetic studies that examine the peak day of suppressor activity were not done and it is possible that CS-A simply shifted the peak day of response. Another reservation concerning this conclusion is that dose response studies to determine the optimum concentration of suppressor cells was not performed. Nevertheless, the findings are suggestive that CS-A is suppressor cell sparing and may even enhance suppressor activity.

Sparing of the suppressor cell population was not adversely affected by increasing concentrations of CS-A as measured by [^{3}H]TdR uptake. We have previously found that 1.0 μg of CS-A per ml will routinely inhibit DNA synthesis up to 90% in both human and *M. speciosa* peripheral blood lymphocytes. However, even this dose was not high enough to inhibit the generation of suppressor cells in these experiments. It is possible that larger doses, i.e., greater than 1.0 μg/ml could be inhibitory.

It is important to note that in these studies CS-A alone was unable to generate suppressor cell activity in peripheral blood lymphocytes. Except for one animal who did significantly suppress mitogenic responses (but not alloantigeneic responses), no other CS-A-cultured cells demonstrated suppressor activity. These findings would disagree with the concept proposed by Tutschka et al. (*10*) who have suggested that CS-A may create an accelerated appearance of suppressor cells. Our experiments indicate that after CS-A treatment, some lymphocytes are spared and continue their physiological immune function, perhaps unaltered. Although we were unable to determine specifically whether CS-A enhanced suppressor cell activity and/or eliminated or inhibited T cell helper populations, our results do support the concepts of Gordon and Singer (*9*). They have performed in vitro experiments with CS-A and have inferred that the mechanism of CS-A may be an inhibition of the T helper cell population (*9*). Deeg et al. (*5*) using an in vivo dog skin graft model were unable to demonstrate a suppressor cell mechanism with MLC in vitro. They postulated that CS-A itself is the "suppressive principle" acting on cell surfaces, perhaps blocking antigen recognition or interfering with cell to cell interactions. Impressively, they were able to demonstrate that CS-A was capable of interfering in vivo with the action of sensitized lymphocytes. They may represent another subpopulation of lymphocytes upon which CS-A is active.

Our laboratory has previously noted that lymphocytes, primed in vitro against specific alloantigens in the presence of CS-A, lose their ability to respond in an accelerated or primed manner to those same alloantigens in a secondary culture. These lymphocytes did, however, respond normally by the 5th day in a secondary culture. These data suggested that CS-A does not induce tolerance by elimination of clones of cells reactive to specific alloantigens. In addition, the constant presence of CS-A was necessary to exhibit its antilymphocytic activity (*7*). Homan et al. (*17*) have reported that CS-A given to rats in doses up to 10 mg/kg for 14 days failed to suppress cell-mediated cytotoxicity (a T cell phenomenon) but did inhibit the humoral responses as measured by lymphocytotoxins appearing in recipient serum. These studies give further evidence that CS-A may exert its immunosuppressive potential on the T helper subpopulations and leave unhindered T suppressor cell activity. If further experiments bear this out, this compound will also prove to be an extremely useful tool in the laboratory to separate T lymphocyte subpopulations.

In conclusion, CS-A seems to spare and in certain instances enhance Con A-generated suppressor cells and their function in the two species examined. This sparing effect was present up to 1.0 μg of CS-A per ml. However, CS-A was unable to induce the generation of suppressor cell activity from peripheral blood lymphocytes without the additional presence of Con A. The immunosuppressive mechanism of this unique compound may result from inhibition or killing of T helper subpopulations and sparing of T suppressor cell fractions resulting in an imbalance of immunoregulation that culminates in profound suppression.

Acknowledgments. We are indebted to Lola J. Levingston for her excellent editorial and secretarial assistance in the preparation of this manuscript. We also thank Dr. Jean Borel from Sandoz, Ltd., Basel, Switzerland, for supplying the CS-A used in these experiments.

LITERATURE CITED

1. WHO Scientific Group on Immunodeficiency: 1979 Clin Immunol Immunopathol 13: 296
2. Moretta L, Webb SR, Grossi CE, et al: 1977 J Exp Med 146: 184
3. Paul WE, Benacerraf B: 1977 Science 195: 1293
4. Borel JF, Feurer C, Gubler HU, et al: 1978 Agents Actions 6: 468
5. Deeg HJ, Storb R, Gerhard-Miller L, et al: 1980 Transplantation 29: 230
6. White DJG, Plumb AM, Pawelec G, et al: 1979 Transplantation 27: 55
7. Leoni P, Garcia RC, Allison AC: 1978 J Clin Lab Immunol 1: 67
8. Leapman SB, Filo RS, Smith EJ, et al: 1980 Transplant Proc 12: 246
9. Gordon MY, Singer JW: 1979 Nature 279: 433
10. Tutschka PJ, Beschorner WE, Allison AC, et al: 1979 Nature 280: 148
11. Borel JF, Feurer C, Magnee C, et al: 1977 Immunology 32: 1017
12. Borel JF, Wiesinger D: 1977 *In* Lucas D (ed) *Regulatory mechanisms in lymphocyte activation*. Academic Press, Inc, New York
13. Shou L, Schwartz SA, Good RA: 1976 J Exp Med 143: 1100
14. Hunninghake GW, Fauci AS: 1977 J Immunol 119: 1122
15. Sakane T, Green I: 1977 J Immunol 119: 1169
16. Tse HY, Dutton RW: 1977 J Exp Med 146: 747
17. Homan WP, Fabre JW, Williams KA, et al: 1980 Transplantation 29: 361

Received 3 June 1980.
Accepted 30 July 1980.

Differential Effects of Cyclosporin-A on Lymphocyte Subpopulations

S. B. Leapman, R. S. Filo, E. J. Smith, and P. G. Smith

THE fungal metabolite, cyclosporin-A (CY-A), is a unique cyclic polypeptide that has potent biologic properties. These activities include a remarkable immunosuppressive effect on lymphocytes without any significant myelotoxicity.[1-3] The suppressive effects of CY-A on lymphocytes may involve only specific lymphocyte populations or subsets of these populations. To elucidate the differential effects of CY-A on normal human peripheral blood lymphocyte subpopulations, the following studies were designed.

MATERIALS AND METHODS

Peripheral blood was obtained by venipuncture from healthy volunteers. Mononuclear cells were separated on Ficoll-Hypaque discontinuous gradients and prepared for culture as described previously.[4] Lymphocytes were then tested for responsiveness by three assay systems: (1) mitogen and alloantigen stimulation, (2) lymphocyte memory by examining primed lymphocyte responsiveness to reference or third party alloantigenic stimulators, and (3) lymphocyte suppression by Con-A induced suppressor cell activity.

Mitogen and Alloantigenic Stimulation

Lymphocytes were cultured in varying concentrations of CY-A. Since CY-A is insoluble in water, it was dissolved in dimethysulfoxide (DMSO). All cells were cultured with 1% DMSO at a concentration of 1×10^5 lymphocytes in round bottom microtiter wells. Mitogen stimulation was carried out with phytohemagglutin (PHA) (1%), concanavalin-A (Con-A) (25 μg/ml), or pokeweed mitogen (PWM) (0.05 μg/ml) for 72 hours.

At the end of this culture period 1 μCi of ^{3}H-TdR was added to each well and the cells were incubated for an additional 12–18 hours. The lymphocytes were harvested onto glass-fiber filter paper with a MASH unit and radioactive incorporation counted in a Packard Scintillation Counter.

The effect of CY-A on alloantigenic responses were examined by culturing responding lymphocytes (1×10^5 cells/well) with irradiated (2500R) alloantigenic stimulators for 5 days in the presence of CY-A. The percent suppression was calculated for both mitogens and alloantigens as follows: percent suppression = (1 − cpm Exp/cpm Control) × 100, where cpm Exp = counts/min CY-A cultured lymphocytes and cpm Control = counts per minute cultured lymphocytes without CY-A.

Memory Cell Function

The effect of CY-A on memory cell activity was examined by studying primed lymphocyte responses to specific or nonspecific alloantigens after those cells had been primed to a specific reference stimulator in the presence of CY-A or hydrocortisone. Details of the memory cell scheme have been reported elsewhere.[4] Briefly, peripheral blood lymphocytes (PBL) were incubated with a specific irradiated stimulator for 10 days in control, CY-A, or hydrocortisone media and then harvested. These primed cells were then stimulated in secondary cultures with either the original alloantigenic stimulator or third party stimulators (Fig. 1). At days 1–5 the cultures were harvested and [^{3}H]TdR incorporation measured in a liquid scintillation counter.

Suppressor Cell Experiments

Suppressor cells were generated by incubating PBL's in media containing Concanavalin-A (10–25 μg/ml) for 72 hours. In some experiments PBL's were cultured in Con-A media plus CY-A (0.1 μg/ml) to determine the effect of CY-A on the generation of Con-A suppressor cells. Control cells were cultured in media containing no Con-A or CY-A. The scheme of the experiments is illustrated in Fig. 2. After three days of culture the primary cells (control, Con-A or Con-A + CY-A) were harvested, irradiated (4000 rads), and added to secondary cultures. Autologous responders in these secondary cultures were stimulated with either alloantigenic irradiated lymphocytes or mitogens (PHA, Con-A, or PWM). These secondary cultures were then incubated for an additional 3 days for mitogen studies or 5 days for alloantigen studies. The cells were harvested and ^{3}H-TdR uptake was

From the Departments of Surgery (Transplantation) and Medicine (Nephrology), Indiana University Medical Center, Indianapolis, Ind.

Supported in part by ONR Contract N-00014-78-C-0412.

Reprint requests should be addressed to Dr. S. B. Leapman, Department of Surgery, Indiana University Medical Center, Indianapolis, Ind. 46202.

0041-1345/81/1301-0091$01.00/0

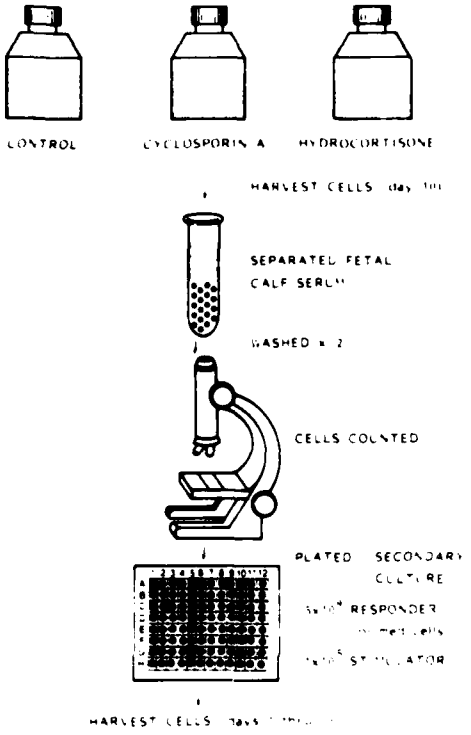


Fig. 1. Scheme of memory cell experiments.

measured. Suppression conferred on these cultures by the putative suppressor cells was then calculated by the following formula: percent suppression = (1 − cpm Exp/cpm NA) × 100. Where cpm Exp = response in counts per minute of secondary culture with Con-A or Con-A + CY-A cells added to the culture and cpm NA = counts per minute of secondary culture with cultured but nonactivated cells added to the culture.

RESULTS

Mitogen and Alloantigen Inhibition by CY-A

Figure 3 shows the dose response curves generated with varying concentrations of CY-A added to the culture media. A 50% inhibitory dose (ID_{50}) of CY-A on mitogen stimulation was found to be 0.31, 0.18, and 0.05 μg for PHA, Con-A, and PWM respectively. The ID_{50} for CY-A on allogeneic responses was 0.25 μg/ml. Pokeweed mitogenic responses were the most sensitive in our series while PHA was the least sensitive to CY-A treatment.

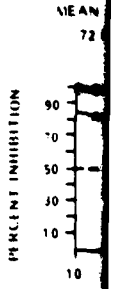
Memory Cell Function

Lymphocytes sensitized to specific alloantigens in either control, CY-A, or hydrocortisone media were assayed for their subsequent primed response to either reference or third party alloantigeneic stimulators in a second culture system. CY-A or hydrocortisone were not present in any secondary cultures. Figure 4 illustrates the primed response of these cells. Control cultures show that the primed cells were stimulated by day 2 with reference alloantigens as expected. This early response was lost in cells primed in the presence of CY-A. However, these lymphocytes were able to respond in a normal MLR fashion to alloantigenic stimulation. This effect on the primed cell response was not observed in hydrocortisone treated primed lymphocytes although these primed cells did respond 24 hours later than did controls.

Suppressor Cell Activity

Mitogenic responses. The suppression conferred on PBL mitogen induced proliferation is shown in Fig. 5. The suppression demonstrated by both Con-A and Con-A + CY-A treated cells was similar for all three stimulatory mitogens. The degree of suppression comparing Con-A to Con-A + CY-A suppressor cells was not significantly different in these experiments. It is important to note that the proliferative responses in these cultures are from responding autologous cells and not from the added suppressor cells since they had been irradiated (4000 rads) prior to their addition to the secondary cultures.

Allogeneic responses. Suppressor cells were added to mixed lymphocyte reactions. Figure 6 demonstrates the suppression induced by the addition of these cells to the MLR. There was a 64% mean suppression induced by Con-A cells compared to 83% by the Con-A + CY-A suppressor cells. These responses were not significantly different



Y-A treat-

ific alloan-
hydrocorti-
subsequent
ce or third
n a second
tisone were
res. Figure
e of these
the primed
reference
ly response
resence of
s were able
fashion to
ect on the
bserved in
mphocytes
espond 24

uppression
proliferа-
uppression
Con-A -
r all three
of suppres-
- CY-A
ly different
int to note
in these
ogous cells
cells since
ds) prior to
ures.
essor cells
reactions.
uppression
ells to the
uppression
to 83% by
ells. These
different

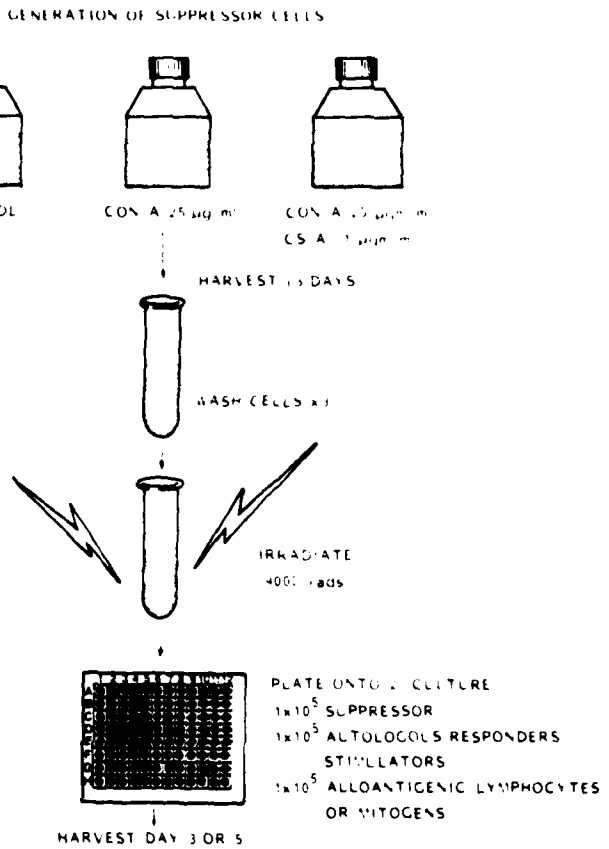


Fig. 2. Scheme of suppressor cell experiments.

indicating CY-A does not effect suppressor cell activity once these cells are generated. Suppression was more pronounced with the more specific allogeneic stimulation than with the more potent but less specific mitogens.

DISCUSSION

Although the specific mechanism of immunosuppression by CY-A remains unclear, several in vivo and in vitro studies have now shown that this compound inhibits thymus

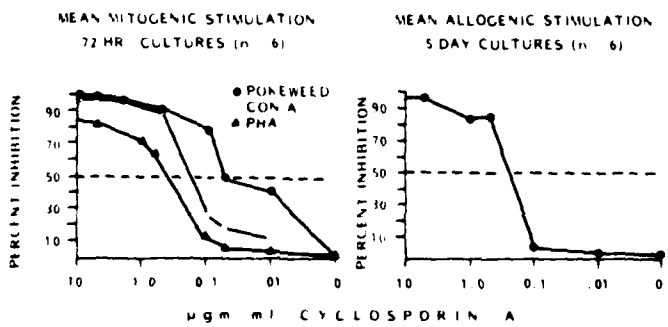


Fig. 3. CY-A dose-response curves of human PBL stimulated with either mitogens or alloantigeneic lymphocytes.

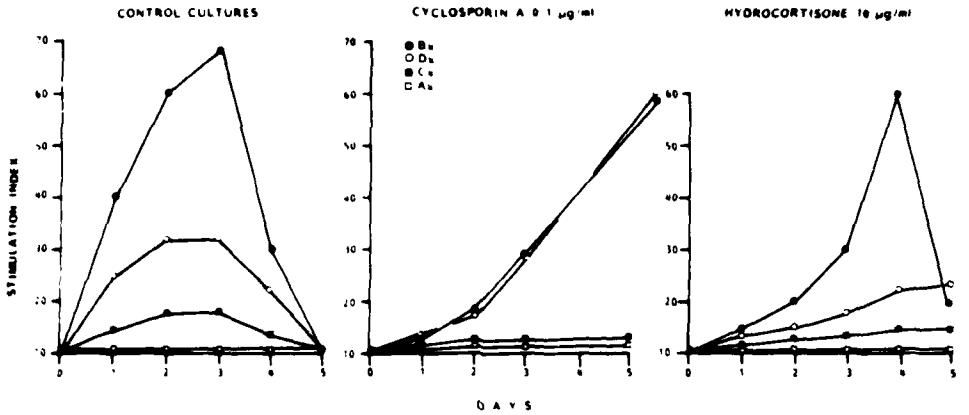


Fig. 4. Response of primed cells (control, CY-A or hydrocortisone) in secondary culture with original stimulator Bx or third party stimulator, Cx, or Dx. Concentrations of CY-A or hydrocortisone were used only in the primary cultures.

dependent (T-cell) humoral and cell mediated immune responses.[3,4] This inhibition, however, seems to be limited to certain subsets of T-lymphocytes, allowing full expression of the unaffected subpopulation.

CY-A is most effective at suppressing lymphocyte proliferation in response to mitogens or alloantigens. This suppression occurs in a dose-dependent fashion to both stimulators. Maximal suppression was seen in Pokeweed cultures where as PHA was the least inhibited. As noted by others, MLR was inhibited by CY-A.[5] It is unknown if this mechanism is a result of disruption of T:T interactions or T:B recognition during the early phase of MLR. In this context it is interesting that CY-A had a definite effect on primed lymphocyte responses in secondary cultures.

These memory cells, primed to respond to a specific alloantigenic stimulator (reference cell) lost this ability to undergo blastogenesis earlier and with greater magnitude if priming occurred in the presence of CY-A. However, these *same cells were able to respond* to the original reference stimulator as well as third party stimulators in a normal MLR manner. Cells primed in hydrocortisone media in doses much greater than CY-A only delayed the

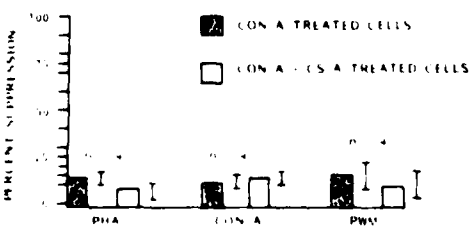


Fig. 5. Suppression of mitogenic stimulation in secondary cultures by the addition of Con A or Con A + CY A pretreated suppressor lymphocytes.

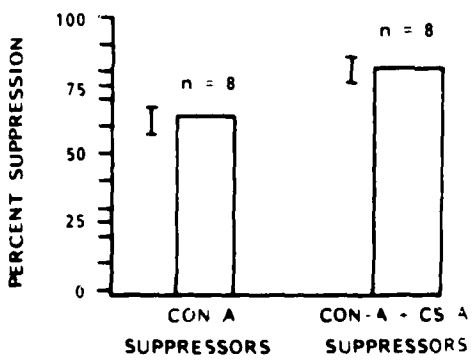


Fig. 6. Suppression of alloantigeneic stimulation in secondary cultures after the addition of Con A or Con A + CY A pretreated suppressor lymphocytes.

primed response by 24 hours. These data would suggest that CY-A interfered with memory cell recognition in the secondary culture but that this loss of recognition was short lived. This temporary loss of "memory" may be a result of T:B interference in the recognition phase of MLR. Also, it would suggest that specific clones of lymphocytes are not eliminated after culture with CY-A.

Reports of suppressor cell sparing by CY-A have recently appeared in the literature.[8-10] Our experiments have demonstrated that the addition of CY-A to Con-A cultured PBL did not effect the generation and subsequent function of suppressor activity in these lymphocytes. This sparing of suppressor cells was present over a wide range of concentrations of CY-A in the primary cultures. Further studies have shown that cells treated only with CY-A do not induce the generation of suppressor cells but rather allow for their expression.

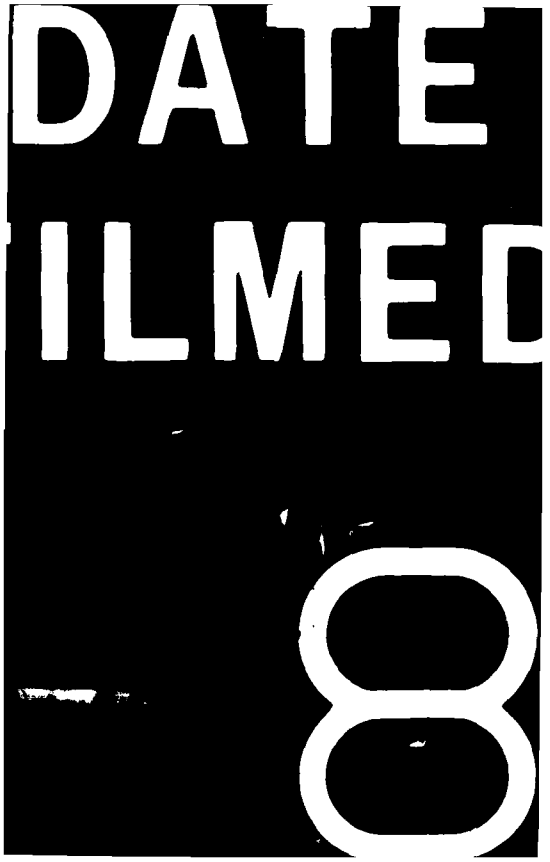
It is not unreasonable to hypothesize that the mechanism of immunosuppression with this unique compound may be a result of inhibition of T-helper subpopulations or interference with T-B or T-T cell interaction exclusive of T-suppressor activity. This would result in an imbalance in immunoregulation which favors suppressor activity and culminates in profound immunosuppression.

ACKNOWLEDGMENT

We are indebted to Lola J. Levingston for her excellent editorial and secretarial assistance in the preparation of this manuscript. We also thank Dr. Jean Borel and Sandoz, Ltd., Basel, Switzerland for providing the cyclosporin-A used in these experiments.

REFERENCES

1. Borel JF, Feurer C, Gubler HU, et al: Agents Actions 6:468, 1976
2. Borel JF: Immunol 31:631, 1976
3. Borel JF, Feurer C, Magnee C: Immunol 32:1017, 1977
4. Leapman SB, Filo RS, Smith EJ, et al: Transplant Proc 12:246, 1980
5. Deeg HJ, Storb R, Gerhard-Miller L, et al: Transplantation 29:230, 1980
6. White DJG, Plumb AM, Pawelec G, et al: Transplantation 27:55, 1979
7. Leoni P, Garcia RC, Allison AC: J Clin Lab Immunol 1:67, 1978
8. Leapman SB, Filo RS, Smith EJ, et al: Transplantation (in press)
9. Hess AD, Tutschka PJ: J Immunol 124:2601, 1980
10. Gordon MY, Singer JW: Nature 279:433, 1979


DATE
ILMED
8